

BD

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 890 650 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:

13.01.1999 Bulletin 1999/02

(51) Int Cl.⁶: **C12Q 1/68**

(21) Application number: **98250244.5**

(22) Date of filing: **02.07.1998**

(84) Designated Contracting States:

**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE**

Designated Extension States:

AL LT LV MK RO SI

(30) Priority: **07.07.1997 JP 195268/97**

(71) Applicant: **Asahi Breweries, Ltd.**
Tokyo 104 (JP)

(72) Inventor: **Sami, Manabu,**
ASAHI BREWERIES, LTD.

Moriya-machi, Kitasoma-gun, Ibaraki (JP)

(74) Representative: **Walter, Wolf-Jürgen et al**

Patentanwälte Felke & Walter

Normannenstrasse 1-2

10367 Berlin (DE)

(54) **Oligonucleotides for detecting lactic acid bacteria**

(57) The present invention provides a method for confirming the presence or absence of a gene resistant to hop, which is a most important factor for determining

beer-spoilage ability of lactic acid bacteria, and for foreseeing rapidly the beer-spoilage of lactic acid bacteria, by using particular oligonucleotides and a judgement method utilizing them.

EP 0 890 650 A2

Description

The present invention relates to oligonucleotides for judging beer-spoilage ability of lactic acid bacteria, which influences beer quality, and a method for judging the beer-spoilage ability with the oligonucleotides.

Beer is effective for controlling bacteria growth, because it contains alcohol, exhausts a carbon source, and has low pH and anaerobic conditions, and further it contains a hop having antibiotic activity. However, since a part of bacteria such as *Lactobacillus* genera is resistant to iso- α -acid, which is an ingredient in the hop, the bacteria mix in beer products and give the products turbidity.

Hitherto, the detection and judgement of bacteria, which influence the quality of beer, have been studied, for example, there are a method for detecting noxious bacteria in beer brewing by using antigen-antibody reaction (Japanese Patent Kokai Publication No. 57-59166), a method for detecting with monoclonal antibody for beer lactic acid bacteria (Japanese Patent Kokai Publication No. 06-105698), and a method comprising extracting DNA from lactic acid bacteria, conducting PCR with primers designated based on a particular sequence, amplifying the sequence and judging the presence or absence of lactic acid bacteria (Japanese Patent Kokai Publication No. 06-141899).

However, these methods are substantially methods for judging particular bacteria such as *Lactobacillus brevis*. For this reason, when *Lactobacillus brevis* not having beer-spoilage ability or the other bacteria having beer-spoilage ability are detected from beer or half-finished goods during beer production, there are problems that the beer-spoilage ability is judged in error, so that the judgement whether detected bacteria have beer-spoilage ability or not is difficult.

The present invention provides a method for judging rapidly whether detected lactic acid bacteria have beer-spoilage ability, by limiting a part of sequence of hop-resistance gene on a plasmid contained in *Lactobacillus brevis* of beer-spoilage lactic acid bacteria (Japanese Patent Kokai Publication No. 8-186621) and the nucleotide in the sequence, and conducting Polymerase Chain Reaction (PCR) with the nucleotide as the primer.

The present invention is an oligonucleotide for judging beer-spoilage ability of lactic acid bacteria, wherein it has a part of the nucleotide sequence or the whole nucleotide sequence described in a sequence listing or has the corresponding complementary sequence. Further, the present invention is an oligonucleotide for judging beer-spoilage ability of lactic acid bacteria, characterized in that a part of the nucleotide sequence is

5'-ATCCGGCGGTGGCAAATCA-3'

or

5'-AATCGCCAATCGTTGGCG-3'.

Preferably, the oligonucleotide of the present invention contains at least 10 continuous bases in above-mentioned nucleotide sequence.

The present invention is further a method for amplifying a target nucleotide, the above nucleotide sequence functioning as a primer of a polymerase chain reaction.

Moreover, the present invention is a method for judging beer-spoilage ability of lactic acid bacteria, said method comprising separating the nucleotide amplified by the above method by using electrophoresis, and judging the presence or absence of the nucleotide having a sequence to be recognized in the resulting nucleotide.

In the present invention, noticing the point that the most important factor for determining the beer-spoilage ability of lactic acid bacteria is hop resistance, since it is able to judge whether detected lactic acid bacteria have a hop-resistance gene or not by using gene sequences relating to the hop resistance, it is possible to foreknow rapidly the beer-spoilage ability of bacteria without confirmation by the bacteria growth in beer and the like.

In the present invention, when lactic acid bacteria is detected from beer or the half-finished goods produced during beer production, the lactic acid bacteria are cultured. When lactic acid bacteria can grow, any culture mediums may be used, for example, the bacteria can increase in a MRS liquid medium. The culture conditions are anaerobic conditions at normal temperature to grow the lactic acid bacteria. The culture times are 1-2 days.

Cultured and increased bacteria are collected, and DNA is extracted by a well-known DNA extract method, for example a method of D.G. Anderson & L. L. Mackay et al. (App. Env. Microbiol. 46, p549, 1983) and is utilized in the judgement of the present invention.

To use two oligonucleotides for using as primers, a part of sequence is determined from the hop-resistance gene described in Japanese Patent Application No. 8-186621, and it is synthesized chemically to obtain two primers opposite to each other. Especially, when the nucleotide sequence described in the sequence listing is used as primers for a polymerase chain reaction, if the lactic acid bacteria have the hop-resistance gene, DNA fragments of 342 bp are

amplified by the judge method of the present invention.

Moreover, thermotolerance at 90°C or more is enough for using the DNA polymerase. The temperature conditions of a PCR reaction are 90-98°C in a thermal denaturation reaction for changing a double strand DNA into a one strand DNA, 37-68°C in annealing of primers to a template DNA, and 50-75°C in a chain elongation reaction to act polymerase.

Using one cycle of these reactions, several tens cycle are conducted to amplify fragments of the hop-resistance gene. After the PCR reaction, the reactant are separated by electrophoresis, nucleic acid stain with ethidium bromide or the like is conducted and the presence or absence of the amplification of DNA fragments is confirmed. When DNA fragments having a chain length specific for the primer designed, it is judged that the bacteria have the hop-resistance gene and beer-spoilage ability.

The present invention is described by working examples in the following. After *Lactobacillus* strains bacteria 95 strains described in Table 1, to be concrete, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus lindneri*, *Lactobacillus collinoides* and the like were cultured in MRS liquid medium for 24 hours, DNA was extracted by a method of Anderson & MacKay.

Using the DNA as a template, and using a part of the nucleotide sequence described in the said sequence list, two primers,

5'-ATCCGGCGGTGGCAAATCA-3'

or

5'-AATCGCCAATCGTTGGCG-3',

PCR was conducted with TAKARA Ex Taq and Program Temp Control System PC-800 (ASTEC).

As to the PCR method, well-known technique may be used (Science 230, p13, 1995).

Five µl of the PCR product was separated by electrophoresis with agarose gel (NuSieve GTG, TAKARA) in which TAE buffer (0.04M, Tris-acetate, 0.01M EDTA, pH8.0) is dissolved to be 3%, and a set of MUPID minigel electrophoresis (ADVANCE CO.), and the PCR product was stained with ethidium bromide. As a molecular weight marker, φX174 DNA (TAKARA) cut with *HincII* was used.

The temperature conditions of the judge method in the present invention are 94°C for 3 minutes as one cycle, 98°C for 30 seconds and 55°C for one minute and 72°C for 0.5 minutes as 25 cycles.

Moreover, as to 95 strains described in Table 1, beer-spoilage ability was tested by the following steps.

Firstly, after culturing in a MRS liquid medium, each bacterium was inoculated to be 10⁵ cells/ml to degassed beer of bitterness value 20, which is adjusted to pH4.6, and cultured at 25°C for two months under anaerobic conditions. After culturing, the strain, which was recognized the presence of beer-spoilage ability by the eye, was judged that it has beer-spoilage ability.

As shown in the results of Table 1, by the judge method (*horA*-PCR) of the present invention, the strains that the specific amplification of 342bp DNA fragments was observed were 61 strains in 95 strains, and the strains that the specific amplification was not observed were 34 strains. In fact, compared with the results that turbidity was recognized after inoculating the bacteria to beer of pH4.6, two strains not having beer-spoilage ability were judged as positive by a *horA*-PCR method, while one strain having beer-spoilage ability was judged as negative by the *horA*-PCR method. Accordingly, it was recognized that the method was very accurate.

Table 1

Bacterium Species	Test Bacterium No.	Judgement by the Present Invention	Beer-spoilage ability
<i>L.brevis</i>	1	+	+
<i>L.brevis</i>	2	+	+
<i>L.brevis</i>	3	+	+
<i>L.brevis</i>	4	+	+
<i>L.brevis</i>	5	+	+
<i>L.brevis</i>	6	+	+
<i>L.brevis</i>	7	+	+
<i>L.brevis</i>	8	+	+
<i>L.brevis</i>	9	+	+
<i>L.brevis</i>	10	+	+

EP 0 890 650 A2

Table 1 (continued)

Bacterium Species	Test Bacterium No.	Judgement by the Present Invention	Beer-spoilage ability
<i>L. brevis</i>	11	+	+
<i>L. brevis</i>	12	+	+
<i>L. brevis</i>	13	+	+
<i>L. brevis</i>	14	+	+
<i>L. brevis</i>	15	+	+
<i>L. brevis</i>	16	+	+
<i>L. brevis</i>	17	+	+
<i>L. brevis</i>	18	+	+
<i>L. brevis</i>	19	+	+
<i>L. brevis</i>	20	+	+
<i>L. brevis</i>	21	+	+
<i>L. brevis</i>	22	+	+
<i>L. brevis</i>	23	+	+
<i>L. brevis</i>	24	+	+
<i>L. brevis</i>	25	-	-
<i>L. brevis</i>	26	-	-
<i>L. brevis</i>	27	-	-
<i>L. brevis</i>	28	-	-
<i>L. brevis</i>	29	+	+
<i>L. brevis</i>	30	+	+
<i>L. brevis</i>	31	+	+
<i>L. brevis</i>	32	+	+
<i>L. brevis</i>	33	+	+
<i>L. brevis</i>	34	+	+
<i>L. brevis</i>	35	+	+
<i>L. brevis</i>	36	+	+
<i>L. brevis</i>	37	+	+
<i>L. brevis</i>	38	+	+
<i>L. brevis</i>	39	+	+
<i>L. brevis</i>	40	+	+
<i>L. brevis</i>	41	+	+
<i>L. brevis</i>	42	+	+
<i>L. brevis</i>	43	+	+
<i>L. brevis</i>	44	-	-
<i>L. brevis</i>	45	+	+
<i>L. brevis</i>	46	+	+
<i>L. brevis</i>	47	+	+
<i>L. casei</i>	48	-	-
<i>L. casei</i>	49	-	-
<i>L. casei</i>	50	-	-
<i>L. casei</i>	51	+	+
<i>L. casei</i>	52	-	-
<i>L. casei</i>	53	-	-
<i>L. casei</i>	54	-	-
<i>L. casei</i>	55	-	-
<i>L. casei</i>	56	-	-
<i>L. casei</i>	57	-	-
<i>L. casei</i>	58	-	-
<i>L. plantarum</i>	59	-	-
<i>L. plantarum</i>	60	-	-

Table 1 (continued)

Bacterium Species	Test Bacterium No.	Judgement by the Present Invention	Beer-spoilage ability
<i>L. plantarum</i>	61	-	-
<i>L. plantarum</i>	62	-	-
<i>L. lindneri</i>	63	+	+
<i>L. lindneri</i>	64	+	+
<i>L. lindneri</i>	65	+	+
<i>L. collinoides</i>	66	-	-
<i>L. collinoides</i>	67	-	-
<i>L. collinoides</i>	68	-	-
<i>L. delbrueckii</i>	69	-	-
<i>L. fermentum</i>	70	-	-
<i>L. rhamnosus</i>	71	-	-
<i>L. sp.</i>	72	-	-
<i>L. sp.</i>	73	-	-
<i>L. sp.</i>	74	-	-
<i>L. sp.</i>	75	-	-
<i>L. sp.</i>	76	+	+
<i>L. sp.</i>	77	+	+
<i>L. sp.</i>	78	+	+
<i>L. sp.</i>	79	+	+
<i>L. sp.</i>	80	+	+
<i>L. sp.</i>	81	+	+
<i>L. sp.</i>	82	+	+
<i>L. sp.</i>	83	+	+
<i>L. sp.</i>	84	+	+
<i>L. sp.</i>	85	-	-
<i>L. sp.</i>	86	-	-
<i>L. sp.</i>	87	-	-
<i>L. sp.</i>	88	-	-
<i>L. sp.</i>	89	+	+
<i>L. sp.</i>	90	+	+
<i>L. sp.</i>	91	+	+
<i>L. sp.</i>	92	+	+

Merits of the invention are as follows. When bacteria having a high possibility of beer-spoilage ability, such as *Lactobacillus brevis* and *Lactobacillus lindneri*, are judged as beer-spoilage bacteria by many conventional identifiable methods, there are many error judges. When beer-spoilage ability is further actually tested by inoculation of bacteria, it takes several weeks to several months.

However, by using the oligonucleotide of the present invention and the method for judging beer-spoilage ability with the oligonucleotide, it is possible to accurately judge the beer-spoilage ability in a few hours.

Annex to the description

SEQUENCE LISTING

5 (1) GENERAL INFORMATION

(i) APPLICANT:

(A) NAME: ASAHI BREWERIES, LTD.,

10 (B) STREET: 7-1, Kyobashi 3-chome

(C) CITY: Chuou-ku

(D) STATE: Tokyo

15 (E) COUNTRY: JAPAN

(F) POSTAL CODE: 104

20 (ii) TITLE OF THE INVENTION: OLIGONUCLEOTIDES FOR
DETECTING LACTIC ACID BACTERIA AND A NEW METHOD FOR
JUDGING BY UTILIZING THEM

25 (iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

(A) NAME:

30 (B) STREET:

(C) CITY:

(D) STATE:

35 (E) COUNTRY:

(F) POSTAL CODE:

40 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:

(B) COMPUTER:

45 (C) OPERATING SYSTEM:

(D) SOFTWARE:

(vi) CURRENT APPLICATION DATA:

50 (A) APPLICATION NUMBER:

(B) FILING DATE:

55 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: Japanese Patent Application No. 9-195268

(B) FILING DATE: July 7, 1997

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Eiko FUNAHASHI

(B) REGISTRATION NUMBER: 100083714

(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 03-3504-0717

(B) TELEFAX: 03-3504-0715

(2) INFORMATION FOR SEQ ID NO.1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 396

(B) TYPE: nucleic acid

(C) STRANDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Plasmid DNA

(vi) ORIGINAL SOURCE

BIOLOGICAL NAME: *Lactobacillus brevis*

SPECIES NAME: AB No.45

(vii) METHOD FOR DETERMINING CHARACTERISTIC: E

(xi) SEQUENCE DESCRIPTION

GGACCATCCG	GCGGTGGCAA	ATCAACCATT	TCTAGCTTAA	TTGAACGTTT	50
TTATGAACCT	AACGAGGGCA	GCATCACGAT	TGGCAATACC	AATATTACTG	100
ATATTCAACT	TGCCGATTGG	CGCCAGCAAA	TCGGCCTGGT	CGGCCAAGAC	150
GCTGCGATCA	TGTCTGGAAC	GATTCGTTAC	AATTTAACCT	ATGGTTTGCC	200
GGGGCATTTT	TCCGATGAAC	AGCTTTGGCA	TGTCTTGGAA	ATGGCTTACG	250
CAACGCAATT	TGTCCAGAAG	ATGCCTCGGG	GCTTGGACAC	GGAAGTCGGT	300
GAGCGTGGAG	TCAAGGTATC	GGGGGGCCAA	CGCCAACGAT	TGGCGATTGC	350
CCGGGCCTTC	CTGCGTAATC	CAAAAATATT	AATGTTGGAT	GAAGCA	396

Claims

1. An oligonucleotide for judging beer-spoilage ability of lactic acid bacteria, wherein it has a part of the nucleotide sequence or the whole nucleotide sequence described in a sequence listing or it has the corresponding complementary sequence.

2. An oligonucleotide for judging beer-spoilage ability of lactic acid bacteria, wherein a part of the nucleotide sequence is

5'-ATCCGGCGGTGGCAAATCA-3'

or

5'-AATCGCCAATCGTTGGCG-3'.

3. An oligonucleotide comprising at least 10 or more continuous bases of the nucleotide described in claim 1 or 2.

4. A method for amplifying a target nucleotide, the nucleotide sequence described in claim 1 or 2 functioning as primers of a polymerase chain reaction.

5. A method of judging beer-spoilage ability of lactic acid bacteria, said method comprising separating the nucleotide amplified by the method described in claim 4 by using electrophoresis, and judging the presence or absence of the oligonucleotide having a sequence to be recognized in the resulting nucleotide.